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INTRODUCTION

Coincidental with the 1939-1945 war came the discovery of several groups of chemical compounds of such extreme toxicity to various insects and plants that their use as insecticides and herbicides for the protection of plants quickly followed their release for peacetime purposes. The better known of these chemicals are: the chlorinated hydrocarbons, a group which includes DDT, lindane and the later members, chlordane, heptachlor, aldrin, and dieldrin; the organophosphorus insecticides, of which the wartime representatives were TEPP, parathion and schradan; the phenoxyacetic acids, of which 2,4-D and MCPA were the earliest members to be used as herbicides.

The commercial success of these new agricultural chemicals encouraged a wide search by industrial organizations for synthetic chemical compounds of equal or better biological activity. This search has been so successful that today the number of known insecticides, fungicides and herbicides is limited mainly by the need for establishing with certainty that their use on crop plants will not have unfavorable consequences on human health. But it was realized that along with the matter of public health, there is the problem of the effects that the long-term and widespread use of these chemicals might have on agriculture itself. Their introduction on the market would generally follow only after successful results of field tests on specific crop plants and against specific pests, diseases or weeds. But the growing crop and the soil which bears it is a complex of living organisms; the introduction to that complex of a new chemical of intense biological activity may have results reaching well beyond the immediate effects for which the chemical was employed. Sometimes the long-term consequences may be serious enough to effect the usefulness of the chemical for its immediate effects. For instance, the widespread use of DDT for fly control was followed surprisingly quickly by the selection of a fly population on which DDT exercised no worthwhile control. Similarly the use of other new insecticides highly toxic to a wide range of insects so reduced the numbers of those insects predatory on phytophagous mites that the latter became serious pests. Sometimes the new chemical had beneficial effects beyond those for which it was used; an illustration of this is the old and well-established practice of partial sterilization of the soil now used as a method of crop and soil improvement, but originally introduced for the control of specific soil-infesting pests and pathogens.

Such are some of the immediate ecological problems raised by the introduction of a new agricultural chemical. But clearly no such chemical can be used to full advantage until it is known how and why it acts on living organisms. Further, for the study of long-term effects, it is necessary to know whether the chemical persists after use, and if not, in what way it is broken down or lost. Hence arises the problem of the biological activity of its decomposition products, and the possibilities that the decomposition products may themselves be useful or detrimental to the grower.

The need for a specialized attack on the variety of problems led to the establishment of this laboratory, and determined the type of work with which it is engaged. It was realized that its problems require an organization in which scientists of the several disciplines involved—chemistry, plant pathology, plant physiology, entomology and bacteriology—could work together in a co-ordinated and unified attack.

Figure 1—Science Service Laboratory, University of Western Ontario, London, Ontario.

This staff has now been in active work for five years and, though this period is brief in relation to the time span of most of the projects under investigation, it is long enough for an appraisal and progress report of its work. The original conception embodied in the above terms of reference has survived. But as the work of the laboratory has progressed, a need for extension has become evident. With higher organisms, a derangement of biological processes is usually fatal; with micro-organisms the effect of the toxicant may be revealed in strange, and as yet little understood, changes in their properties. The variation in growth habits produced in many fungi and bacteria by the changes in their nutrition is but one of a group of phenomena generally described by the non-committal term of dissociation. Moreover there is now a large body of evidence to indicate that the interplay of the soil microflora or fauna—even the relationship between crop plant and pathogen or pest—is controlled or influenced by metabolic products of high biological potency produced by the organisms themselves. An example, the practical significance of which is still not clearly established, is the production by soil fungi of anti-fungal compounds of the general group labelled antibiotics. It is apparent that a knowledge of the effects of exotic agricultural chemicals upon the biological environment of the crop plant cannot be complete until the nature and properties of the biologically-active chemicals produced normally in that environment are known. To this latter subject, which previously has received only fragmentary or unco-ordinated study, the term "ecological chemistry" has been applied.

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FUNGICIDES

The Dithiocarbamates and Related Fungicides

The fungicidal properties of the dithiocarbamates were first discovered in the early 1930's, a discovery protected in 1934 by U.S. Patent 1,972,961, in which it was stated that all compounds containing the group >N-CS-S- "are of value for the control of fungi of various kinds". The introduction of these compounds into agricultural practice was facilitated by their prior use as accelerators in rubber vulcanization, and it is not surprising that those already in production for this purpose should be the first to be put to field tests as fungicides. The outcome is that the ferric and zinc salts of dimethyldithiocarbamic acid, christened ferbam and ziram respectively, are standard fungicides used mainly as foliage protectants, and that tetramethylthiuram disulphide, with the common name thiram, is widely used as a non-mercurial seed dressing and as a soil fungicide.

In 1943 it was discovered that the sodium salt of ethylenebisdithiocarbamic acid, though readily soluble in water, was a protective fungicide. It would therefore appear that this compound, since called nabam, decomposes after spraying to form a water-insoluble and fungicidal residue. In field treatments nabam gave erratic results and was eventually displaced by the zinc and manganese salts of ethylenebisdithiocarbamic acid, which have received the names zineb and maneb respectively. These metallic salts make up the greater part of the two million pounds of dithiocarbamates sold annually in recent years in Canada for pest control purposes. This consumption is on a par with that of the wettable sulphurs and of copper sulphate. In spite of this wide use, little was known in 1950 of the reactions by way of which these compounds became fungicidal.

Nabam:

Attention was first directed to nabam, and to the intriguing problem of its decomposition to a spray deposit effective as a protective fungicide.

Aeration of dilute solutions of nabam gave rise to a yellow insoluble product which was shown to contain three fungicidal components, namely, elementary sulphur in amounts up to 15 per cent, a compound new to science and identified as ethylene thiuram monosulphide (I) in amounts ranging from 10-20 per cent and a highly water-insoluble material which, from its analysis and behavior, is thought to be polyethylene thiuram monosulphide. The abilities of both the monomeric and polymeric forms of (I) to inhibit the germination of spores of *Sclerotinia fructicola* (Wint.) Rehm were high enough to account fully for the fungicidal properties of the nabam deposit (13, 21 and 32).

So far, attempts to produce compound (I) (hexahydro-1,3,6-thiadiazepine-2,7-dithione) by an unambiguous synthesis have failed. The evidence in favor of the proposed structure is based on its ultraviolet and infrared spectra, and on its reaction with ammonia, which yields ethylenedithiobiuret (hexahydro-1,3,5-triazepine-2,4-dithione).

The manganese salt of ethylene bisdithiocarbamic acid is appreciably water-soluble, and aeration of its solution also leads to the formation of ethylene thiuram monosulphide. Indeed, ethylene thiuram monosulphide was identified as a component of a two-year-old sample of commercial maneb.

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Zineb, on the other hand, is highly insoluble in water, and on aeration its suspension does not produce ethylene thiuram monosulphide unless the suspension is made alkaline. The addition of sodium hydroxide greatly enhanced the fungicidal activity of those samples of zineb which are stable on aeration. Moreover, the fungicidal activity of ethylene thiuram monosulphide was increased by the addition of some small amounts of zinc hydroxide.

It is well known that under acid conditions nabam decomposes with the formation of ethylene thiourea, hydrogen sulphide and carbon disulphide. The standard analytical methods depend on the estimation of the amount of carbon disulphide so produced. As these products of acid decomposition have a low and, for practical purposes, insignificant fungicidal activity, it is important to determine whether this type of decomposition can occur under field conditions.

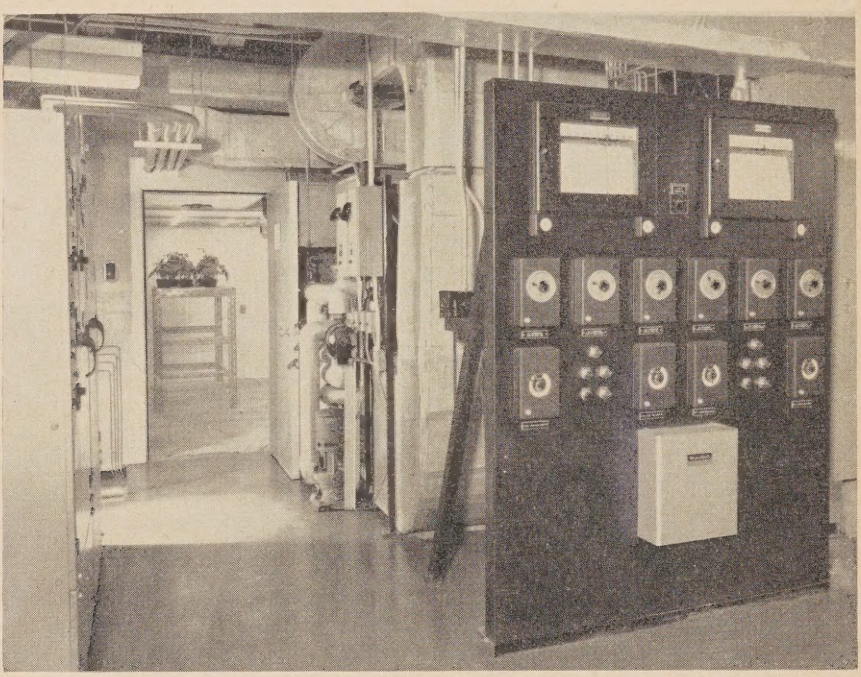


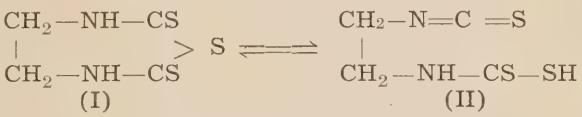
Figure 2—Plant Growth Rooms—a section showing one growth chamber and its control machinery.

Plants sprayed with nabam and held in closed chambers in the dark were severely injured, whereas plants similarly sprayed but not held in the dark remained healthy. The damage produced was similar to that caused by an accumulation of carbon disulphide and hydrogen sulphide. This presumably occurred through the acid decomposition of nabam due to the accumulation of respiratory carbon dioxide in the water film on the leaves of the darkened plants—an accumulation prevented by photosynthesis on the plants exposed to light. The possibility that a comparatively slight decrease in the pH conditions of the leaf surface may shift the decomposition of nabam from the fungicidal ethylene thiuram monosulphide route to the non-fungicidal and phytotoxic carbon disulphide hydrogen sulphide route may explain the inconsistent field performance of nabam as a protective fungicide. Moreover the stability of zineb and its conversion to an effective fungicide in the presence of a small amount of alkali provide an explanation of the erratic results experienced in its field use. A further outcome is of significance in the analysis of zineb and maneb, and in the estimation of spray residues of nabam and its metallic salts. As mentioned above, the analytical method relies on the amount

of carbon disulphide produced by acid decomposition. But ethylene thiuram monosulphide and its polymer do not yield quantitative amounts of carbon disulphide on acid digestion, and this method may therefore yield fictitiously low results.

But the most significant of the practical applications of this work is that a direct use of ethylene thiuram monosulphide or its polymer would bypass the reactions responsible for the erratic field performance of nabam, zineb and maneb. A material closely akin in properties to that produced by the aeration of dilute nabam solutions has been produced commercially under the name "thioneb". This product has been subjected to field trial alongside standard fungicides and the material produced in the laboratory by the aeration of spray-strength nabam solution. In greenhouse and field tests carried out by the courtesy of Dr. J. M. Hamilton of the New York Agricultural Experimental Station at Geneva, thioneb and ethylene thiuram monosulphide both controlled apple scab and the cedar rust fungus. At the Science Service Laboratory at St. Catharines, the laboratory product and the one commercially produced gave results superior to standard fungicides in the orchard control of both the blossom blight and fruit rot stages of brown rot of peach. Results of tests here and at the Rust Research Laboratory, Winnipeg, on the control, in the greenhouse, of wheat stem rust with the two products were most promising, but subsequent field trials have not given encouragement.

Workers at the Institute for Organic Chemistry at Utrecht demonstrated the high fungicidal activity of ethylene diisothiocyanate, and showed that a range of fungi displayed identical susceptibility to this compound and to the ethylenebisdithiocarbamates. They suggested that the anti-fungal action of nabam is due to the formation *in situ* of ethylene diisothiocyanate. The instability of the latter compound renders this hypothesis unattractive but, on confirming our report of isolation of ethylene thiuram monosulphide, the Dutch workers suggested that ethylene thiuram monosulphide exists in solution in equilibrium with an isomeric open compound containing the isothiocyanate group.



(I) (II)

Evidence in support of this pleasing compromise between the views of the Dutch school and ourselves was eventually found by infrared spectroscopy. Because of the instability expected of the isothiocyanate (II) in aqueous solution, non-aqueous solvents were used. Anhydrous ferric sulphate was added to a chloroform solution of ethylene thiuram monosulphide, and the absorption bands (1735, 2130, 2210 cm^{-1}) characteristic of the isothiocyanate grouping appeared (35).

The reactions by which nabam becomes fungicidal, therefore, would seem to follow the route: nabam → ethylene thiuram monosulphide → isothiocyanate. It remains to be determined how the isothiocyanate group inhibits fungal growth. A clue to the vital process involved is given by the observation that the fungicidal activity of nabam or ethylene thiuram monosulphide is reduced by the addition of thiol compounds such as cysteine, thioglycollic acid or glutathione. A reaction with sulphydryl enzymes is therefore suspected, and it has been shown that ethylene thiuram monosulphide (and also thiram) at 10^{-5} molar, or nabam at 10^{-4} molar, inhibits to an appreciable extent the enzymes glucose-6-phosphate and 6-phosphogluconate dehydrogenases. The dithiocarbamates may therefore block the hexose monophosphate oxidation pathway.

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Thiram:

The extensive use of thiram (tetramethylthiuram disulphide) as a seed protectant and soil fungicide, and particularly its recommendation for the control of sugar beet root rot, raises the question of its fate and persistence in soil. The first problem was to perfect a method of bioassay of thiram in soil. The ethanol extract of the air-dried soil was placed on a disk of filter paper and, after draining, the disk was placed on solid peptone agar in which spores of *Glomerella cingulata* (Stonem.) Spauld. & Schrenk. were uniformly dispersed. On incubation the diameter of the clear zone in which germination of the spores had been inhibited around the disk was measured. The diameter of the inhibition zone was found, under the conditions of the experiment, to be directly proportional to the log concentration of the fungicide. By this technique a rapid disappearance of thiram from the soil was revealed and, because the amount persisting is not in logarithmic relation to time, it is suspected that more than one process is involved. As sterilization of the soil by heat or by propylene oxide did not prevent a rapid disappearance of thiram from the soil, the process may proceed independently of biological factors. Soil perfusion methods, however, showed that microorganisms may be involved; viable fungus spores and yeast spores bring about its disappearance from solution at a rate dependent on the relative concentration of spores and thiram. The first step of the microbiological degradation is reduction to the dimethyldithiocarbamate ion, which exists in equilibrium with thiram. The study of this oxidation-reduction relationship is in hand (25).

In the course of synthetic work preparatory to the incorporation of radiosulphur in thiram, a previously undescribed reaction between thiram and acetone was observed. It was learned by correspondence that this reaction was also being examined at the B. F. Goodrich Research Center, and a joint publication resulted. Dimethylamine is an intermediate in the reaction, from which it was concluded that any compound such as acetone, having an active hydrogen, would react with thiram to produce a dithiocarbamate derivative. The reaction thus provides an alternative route for the *in vivo* production of dithiocarbamate from thiram, and may have significance in the fungicidal action of thiram (56).

The protection of seedlings from *Pythium ultimum* Trow afforded by thiram lasts long after the thiram content of the soil has been reduced below the level toxic to the pathogen. Thiram treatment was found to depress the growth of most soil fungi, but to encourage the development of many bacteria and certain saprophytic fungi, including *Trichoderma* and *Penicillium*. This selective action was also revealed in the study of the effect of percolate from thiram-treated soil; inhibition of the growth of *P. ultimum* was obtained with percolates even when the thiram content was below toxic concentrations. Two factors may therefore cause the suppression of the pathogenic fungus subsequent to thiram treatment: first, competition by resistant saprophytic microorganisms; second, the toxicity of anti-fungal substances produced by the latter. *T. viride* (Pers.) Fr., for example, is known to produce in artificial culture the fungicidal gliotoxin and viridin, both antibiotics known to be capable of protecting seedlings from damping-off fungi (25).

Copper 8-quinolinolate

Copper 8-quinolinolate has high but anomalous anti-fungal activity, for not only is it highly insoluble in water but the fungicidal activity of 8-quinolinol is usually attributed to its ability to form stable chelates with metals present in certain enzymes. The problem of the synthesis of related compounds was therefore given for postgraduate study. The quinoline derivatives

prepared and examined included 2-quinolinol, 2-methyl-8-quinolinol, quinaldic acid, quinoline 8-carboxylic acid, 8-hydroxyquinaldic acid and 8-methoxy-2-methyl-quinoline; the pyridine derivatives included picolinic acid, 2-hydroxypyridine, 5-methyl-2-hydroxypyridine and 2-pyridine-methanol. Only 8-quinolinol and 2-methyl-8-quinolinol and their copper chelates were appreciably toxic to spores of *Sclerotinia fructicola;* in the series tested the requirements for fungicidal activity were a quinoline nucleus and an ability to chelate to form compounds of high lipoid solubility.

Alkylated Ethylene Thioureas

A well-tried device in the investigation of the reasons for biological activity in a given compound is the comparative examination of its homologues. In the alkyl dithiocarbamates, for example, fungicidal activity is greater the smaller the alkyl group; in the alkyl imidazolines (B.P. 598,927) fungicidal activity is greatest at the heptadecyl ($—C_{17}H_{36}$) member, the acetate of which is glyodin; the most phytotoxic of the series appears around the C_{13} member. Examples of this type provide verification of Ferguson's rule and are explainable on the solubility relationships.

A comparative study of fungitoxic-phytotoxic relationships on the lines illustrated by the above example of the imidazolines is a promising route to the discovery of systemic fungicides. For this reason, and to provide for an extension of Ferguson's rule, the homologous series of N-*n*-alkyl ethylene thioureas (1-alkyl-2-imidazolidinethiones) was synthesized (51).

Fungicidal activity increases as the series is ascended to the octyl member and then declines. When applied to seed or to the roots of plants growing in sand culture, phytotoxicity is greatest at the amyl and hexyl members. Water solubility, as expected, decreased with increase in length of the alkyl group, whereas the partition coefficient oil/water increased as the series is ascended. The results conform to the general case, but an interesting extension was provided by the observation that, when phytotoxicity was tested by using cut shoots or tissue sections, greatest activity was shown, not by the amyl homologue, but by the octyl member. The high oil/water coefficient of the octyl member is reflected in the powerful absorption, which permits fungicidal activity but which prevents the chemical from entering through intact plant roots; N-*n*-amyl ethylene thiourea has a lipoid/water coefficient which permits entry through the intact roots and the reaction within the plant cell which results is phytotoxicity.

Miscellaneous Problems in Plant Pathology

In spite of the vigorous control measures, shipments of tulip bulbs imported into western Ontario are often intercepted because of infection by *Botrytis tulipae*. Though the average infection is below 2 per cent it is a potential source of primary infection. The question was raised whether or not a fungicidal treatment of the bulbs was a feasible method of reducing this hazard. Preliminary tests showed that standard fungicides such as thiram, chloranil and sodium *o*-phenylphenate at economical concentrations inhibited *in vitro* the growth of *B. tulipae* (Lib.) Lind. Infected bulbs were accordingly dipped in suspensions of fungicides at appropriate concentrations and times, but these treatments did not prevent the development of the disease. This failure to control the disease is probably due to the inability of the fungicide to penetrate the outer bulb scales and to reach the deep-seated mycelium of the causal fungus (16).

The potential uses of fungicidal fumigants have never yet been widely explored, nor experimental techniques developed. For the latter reason, the application to fumigants of the now classical methods of probit analysis has been studied, for this technique has greatly enriched knowledge of the mode of action of foliage fungicides. Previous test methods have, with few exceptions, involved the treatment of the fungus on its natural substrate, on agar or on impregnated cloth, introducing the complication of substrate penetration by the fumigant. For the present study spores of the fungus under test were treated on sintered glass disks, and their germination density was observed. Good regression lines of probit-mortality on the logarithm of the dosage were obtained with both ethylene oxide and chloropicrin, and the great influence of relative humidity on the regression constants was revealed (59).

INSECT TOXICOLOGY

Physiology and Biochemistry of Insects

To provide a basis for understanding how insecticides interfere with the vital processes of insects, the laboratory has devoted considerable attention to filling the numerous gaps in our knowledge of the normal physiology and biochemistry of insects. Enzyme inhibition is so plausible an explanation of toxicity that a knowledge of insect enzymology seems a prerequisite to the study of insect toxicology. The multi-enzyme process of carbohydrate metabolism affords many points where inhibition of enzymes could interrupt the vital mechanisms for energy production. Studies on carbohydrate metabolism were therefore undertaken and are outlined below. Another natural point of departure was afforded by the hypothesis that the lethal effect of the organophosphorus insecticides results from inhibition of the nerve enzyme, cholinesterase. Studies were initiated, therefore, to determine whether this hypothesis, developed for vertebrates, could be invoked also for insects. Dysfunction of the nervous system of insects appears to follow poisoning with many insecticides, and electro-physiological studies have recently been introduced into the laboratory program to afford information on the normal physiology of nervous activity and the changes following exposure to insecticides.

(*a*) *Studies on acetylcholine in insects:*

In vertebrates, death following exposure to organophosphorus compounds has been explained by the accumulation of acetylcholine, which is produced at nerve endings and is responsible for the transmission of nerve impulses across synapses. Normally, acetylcholine is almost instantaneously hydrolysed to inactive compounds by the action of cholinesterase. The organophosphorus poison inhibits the enzymic action of cholinesterase and thereby produces an accumulation of acetylcholine, which results in a disruption of the normal synaptic transmission of the nerve impulses.

The use of this process as an explanation of the insecticidal action of these compounds was tempting, but remained hazardous so long as it was uncertain whether acetylcholine occurs in the insect body, and whether its formation, functions and enzymic hydrolysis follow the same patterns in insects as in vertebrates. These deficiencies in knowledge of the physiology of nervous function in insects had clearly to be remedied.

A first step was to determine that acetylcholine is present in insects. A major difficulty was overcome when it was found possible to separate the positively-charged acetylcholine from interfering substances in housefly head homogenates by the technique of paper electrophoresis. After this preliminary

separation acetylcholine was identified by paper chromatography, by the Hestrin chemical test, by pharmacological assay and by infrared spectroscopy. Moreover, only one substance was present in the extract which had the characteristic biological reaction of causing the contraction of the eserinised frog's rectus abdominis muscle, and this substance proved to be acetylcholine (43, 70).

Having shown acetylcholine to be present in insect tissue, the next problem was to determine the amount normally present. The development of methods for the estimation of acetylcholine proved difficult because of its extremely rapid hydrolysis during the extraction procedure. For instance, a rapid freezing of the insect tissue in liquid nitrogen failed to preserve the initial content because of enzymic hydrolysis at the moment of thawing, even when the frozen tissue was intimately dispersed in a freezing slush of trichloroacetic acid. High recoveries were finally obtained by a method in which the excised insect tissues were immediately boiled and the subsequent extraction procedure was modified to reduce manipulative loss. The results confirmed earlier evidence that the acetylcholine content of insect nervous tissue is much higher than that of vertebrate nervous tissue. This work was done in collaboration with S. E. Lewis, Pest Infestation Laboratory, D.S.I.R.

If the organophosphorus insecticides are toxic because they prevent the hydrolysis of acetylcholine, the evidence that the latter compound is comparatively harmless to insects when injected into the body cavity requires an explanation. A method was accordingly devised for the study of the rates of hydrolysis of various esters which were added to the fluid in which a roach preparation was placed. For the latter a roach, anæsthetized with carbon dioxide, was opened by a single dorsal cut, and the integument deflected and pinned back in a manner which preserved intact as far as possible the membranes surrounding the nerve cord, fat body and other important tissues. The roach was placed in a bath permitting a continuous irrigation of tissues with aerated Ringer's solution to which was added the ester under study. The hydrolysis of the ester was followed by colorimetric or electrometric methods. Phenyl acetate, *o*-nitrophenyl acetate and triacetin were hydrolyzed promptly and readily, whereas acetylcholine and other ionizable esters were not hydrolyzed. It is suggested that there is, in the roach, a barrier impervious to ions, which prevents contact by the esterase and substrate. In support of this hypothesis a relationship was found between the potency and the pK of various nitrogenous bases and quaternary salts known to be toxic to mammals (71).

(*b*) *Studies on choline acetylase in insects:*

As it was known from published work that cholinesterase, the enzyme responsible for the hydrolysis of acetylcholine in vertebrates, is present in insects, the next step was to demonstrate that choline acetylase, the vertebrate enzyme involved in the synthesis of acetylcholine, is also present. By the use of acetone powders of blowfly heads, not only was the presence of the enzyme demonstrated, but also the enzymic system necessary for the production of its substrate, acetylCoA. AcetylCoA is produced by a process which utilizes the energy derived from ATP and in which acetate or citrate may serve as the acetyl donor; the acetyl group of acetylCoA is then transferred to choline by the terminal enzyme choline acetylase to produce acetylcholine. A method of separating these two reactions was devised, thus permitting the activity of choline acetylase to be measured independently of the rate of acetylCoA formation. By this method it was shown that the activity of choline acetylase in preparations of blowfly heads is much higher than it is in the mammalian brain. In the latter tissue it was shown that choline acetylase is associated with mitochondria of the nerve cells—a finding of much physiological interest,

for recent work has shown that there is a high concentration of mitochondria in the synapses of the central nervous system. This experience has been gained by work on the mammalian brain, and it will now be applied to investigate the intracellular distribution of the enzymes in insect nervous tissue.

The work summarized in the above paragraph was carried out at the National Institute for Medical Research under Dr. W. S. Feldberg, F.R.S., and at the Institute for Animal Physiology, Cambridge, in collaboration with Dr. C. O. Hebb. This transfer of work was made possible by the courtesy of the Directors of these Institutes.

(*c*) *Studies on insect cholinesterase:*

Having shown that acetylcholine is present in insects and that its manufacture is by a process similar to that in mammals, it remains to determine whether the insect enzyme responsible for the destruction of acetylcholine is akin to the cholinesterase of vertebrates. The substrate specificity and kinetic properties of the insect enzyme are similar to those of "true" cholinesterase. The insect enzyme was found to be distributed, *in vitro*, between a soluble fraction remaining unsedimented after centrifugation at 50,000 times gravity, and a particulate fraction which is thrown down at low gravitational forces at which only large cell fragments and nuclei would be precipitated. The proportion of the enzyme remaining in the soluble and particulate fractions, when the insect tissue was homogenized, was found to be determined both by the pH and the electrolyte concentration of the homogenate (38, 39).

Because this finding is of interest in connection with intracellular distribution of cholinesterase in insects, a matter bearing on the properties of the organophosphate insecticides and other cholinesterase inhibitors of potential insecticidal use, the effects of electrolytes on the activity of the enzyme were examined further. The standard methods of assessing cholinesterase activity are the titrimetric method, in which the acetic acid liberated by the hydrolysis of acetylcholine is determined by titration, and the manometric method, in which the acetic acid is determined by the amount of carbon dioxide evolved in the presence of sodium bicarbonate. It was found that simple electrolytes such as sodium chloride activate the enzyme, and that the former method gave estimates of the activity of the enzyme two or three times greater than those estimated by the manometric method. The difference was traced to an inhibitory action of the bicarbonate ion, and to the lower basal activity obtained with the titrimetric method in the absence of salts. These observations are of importance in the quantitative examination of cholinesterase activity and of its inhibition by insecticides (37).

For the study of the substrate specificity of insect cholinesterase the following compounds were prepared:—*O*-acetyl-, *N*-acetyl-, *O,N*-diacetyl- and *O,N,N*-triacetyl-tyramine, acetylhordenine hydroiodide and methiodide. The *O*-acetylated compounds were rapidly hydrolyzed by either mammal or flyhead cholinesterase, *O*-acetyltyramine at a rate faster than acetylcholine by the insect cholinesterase. The enzyme responsible was shown by tests with a range of competitive and non-competitive inhibitors to be cholinesterase, and not a general esterase.

It has been shown that ethanol, itself an inhibitor of cholinesterase, also protects the enzyme from organophosphorus inhibition. This effect may account for a misleading interpretation of results obtained with ethanolic solutions of water-insoluble organophosphorus compounds, in which the effects of solvent have been corrected by reference to an alcohol-containing control.

Some doubt concerning the ubiquity of cholinesterase was raised when the enzyme was reported to be absent in *Tribolium* and *Tenebrio*. The apparent absence of the enzyme in these insects was shown to be an artifact resulting

from the preparative method and its presence has now been demonstrated by the use of conventional methods (12).

(*d*) *Glycolysis in insects:*

It was established that glycolysis in the adult housefly is of the phosphorylated type, for all the phosphorylated intermediates of the Embden-Meyerhoff system are metabolized by housefly homogenates. The following glycolytic enzymes were found to be active: hexokinase, phosphofructokinase, phosphohexose, isomerase, aldolase, *a*-glyceraphosphate dehydrogenase, triosephosphate isomerase, triosephosphate dehydrogenase, enolase and lactic acid dehydrogenase. A high rate of phosphorylation is possible only in the presence of hexose diphosphate, necessary presumably as a reservoir of organic phosphate. Moreover, the addition of inhibitors was necessary to prevent degradative reactions which would tend to decrease the level of organic phosphate. The glycolytic enzymes are not associated with the mitochondria but are either soluble or associated with particles which remain unsedimented after centrifugation at 12,000 x g for 10 minutes. Triosephosphate dehydrogenase was active only in the presence of sulphydryl compounds, such as cysteine or reduced glutathione.

Several interesting differences were noted between the insect and the mammalian glycolytic enzymes. Insect *a*-glycerophosphate dehydrogenase is capable of oxidizing *a*-glycerophosphate either with TPN or DPN, whereas the mammalian enzyme isolated from muscle appears to be active only when in the presence of DPN. Insect triosephosphate dehydrogenase was apparently insensitive to iodoacetate at 10^{-4} molar (31).

To supplement the above-described direct observational work, the possibility of an identification of the intermediates by chromatographic methods was explored. If, for example, the insecticide was capable of enzyme inhibition, this inhibition might be revealed by an accumulation of the normal substrate of the affected enzyme. A clear-cut separation of the phosphate sugars proved difficult. By a preliminary separation of a trichloroacetic acid extract of the esters into soluble and insoluble barium derivatives, the components of the barium-insoluble fraction were separated in appropriate solvents. The Krebs cycle intermediates could be separated by two-dimensional chromatography.

(*e*) *Hexose monophosphate oxidation:*

Alternative to the Embden-Meyerhoff anaerobic scheme for the degradation of glucose, an aerobic pathway is known in plants and higher animals. This route was found in the flight muscle of the housefly. At least seven enzymes are involved: glucose-6-phosphate dehydrogenase, gluconolactonase, 6-phosphogluconic dehydrogenase, phosphoketopentose epimerase, phosphopentose isomerase, transketolase and transaldolase. The stoichiometry of the several reactions indicates that glucose-6-phosphate is oxidized to phosphogluconolactone. The latter is then enzymically hydrolyzed to 6-phosphogluconic acid, which in turn is oxidized to a ketopentose. This ketopentose fraction consists of ribulose and xylulose. Two moles of pentose phosphate are then converted to heptulose- and triose- phosphates. The heptulose phosphate reacts further to form hexose phosphates (52).

The glucose-6-phosphate and the 6-phosphogluconic dehydrogenases involved in the above route are located in the cytoplasm. Both enzymes are activated by manganous, magnesium and potassium ions; the former is activated by cobaltous ions which inhibit the latter; both are inhibited strongly by mercuric and zinc ions. The pH optium for glucose-6-phosphate dehydrogenase is 9·0-10·0; that for 6-phosphogluconic dehydrogenase is 7·5. Both enzymes require triphosphopyridine nucleotide, no activity being detected in the presence of diphosphopyridine nucleotide. A similar specificity was also

found for extracts of the peach aphid and pea aphid. Both are sulphydryl enzymes inhibited by *p*-chloromercuribenzoate or iodosobenzoate and are found in the head, thorax and abdomen of the housefly. The abdomen contains the highest activity (75).

(*f*) *Electrophysiological studies:*

To support investigations on the mode of action of insecticides, studies were initiated into the neurophysiological mechanisms and pathways of insect nervous systems. Basic apparatus for these studies include an oscilloscope, pre-amplifier, square wave stimulator and a high speed camera. An electrode manipulator, a pipette puller, and an automatic air puffer have been designed for specific projects.

To show the pathway of nervous conduction in the central nervous system of *Periplaneta americana* L. sensillae on the cerci were stimulated by air. Adaptation to the stimulus occurred in the intact insect but not in the ventral cord of the isolated abdomen. The thoracic ganglia took part in shunting the stimulus to the leg nerves.

This pathway has been of use in the study of the role of acetylcholine in relation to nervous activity. Stimulation of the cercal sensillae by puffs of air resulted in an increase of acetylcholine much above the normal level in eserinized thoracic ganglia. Electrical stimulation of the ventral cord produced a similar result. It is concluded that the release of acetylcholine is related to nervous activity in the thoracic ganglia, and it is presumed that acetylcholine is a synaptic transmitter.

INSECTICIDES

(*a*) *Effect of insecticides on various metabolic enzymes:*

No inhibitory effect on glucose-6-phosphate dehydrogenase or on 6-phosphogluconate dehydrogenase was shown by concentrations of 10^{-4} molar of the following insecticides: (*a*) the organophosphorus compounds: malathion TEPP, schradan, dimethyl trichlorohydroxyethyl phosphonate ("Dipterex"); (*b*) the insecticidal carbamates: dimethyldihydroresorcinol dimethyl carbamate ("Dimetan") or methylphenylpyrazolyl dimethylcarbamate ("Pyrolan"); (*c*) the chlorinated hydrocarbons: DDT, lindane, γ-chlordane, aldrin, dieldrin, heptachlor, methoxychlor, isodrin and endrin.

In one of the earliest attempts to establish the mode of action of DDT it was shown that the insecticide had no effect on the cholinesterase of insects, *in vivo* or *in vitro*. A later report claimed that under certain conditions, a strong inhibition of the enzyme could be demonstrated. Repeated attempts at this laboratory have failed to confirm this finding. Neither DDT nor aldrin had a significant effect on the activity of the enzyme from flies or roaches, *in vivo* or *in vitro*.

(*b*) *Schradan:*

Schradan was the first of the organophosphorus compounds found to have systemic insecticidal properties. Plants watered or sprayed with dilute solutions of schradan become toxic to sap-feeding insects. Yet the explanation that these insecticidal properties arise through an inhibition of insect cholinesterase presents difficulty, for schradan itself is not an effective inhibitor of the enzyme when tested by *in vitro* methods. Moreover, plants treated with schradan are not effectively toxic to leaf-eating insects, yet sap-feeding insects are killed by an anticholinesterase action. Two questions at once emerge:— Is schradan converted by the insect to an effective anticholinesterase and by what process? Why is schradan effective as an insecticide only against sap-feeding insects?

It was known that the inhibition of cholinesterase by organophosphorus compounds is due to a phosphorylation of the enzyme, an ability which is correlated with the ease of hydrolysis of the phosphorus compound. Presumably therefore schradan, which is stable in an aqueous solution, must be converted to compounds more readily hydrolyzable before it can be insecticidal. To test this hypothesis schradan was subjected to chlorination. Kinetic studies revealed the production of mono-, di-, tri- and tetra-chlorinated derivatives. Monochlorinated schradan showed in *in vitro* tests an activity as an anticholinesterase some 100,000 times that of schradan itself. Moreover, in aqueous solution at pH 9 and room temperature, the monochlorinated compound had a half-life of 40 minutes as compared with over two years for schradan itself. The hydrolysis of the more highly chlorinated schradans was so rapid that they decomposed before they could react with the enzyme in tests for anticholinesterase activity (19).

It having been shown that schradan could be "activated" by the introduction of an electrophilic substituent such as chlorine, the next step was to compare the anticholinesterase with that likely to be produced biologically. As the activation by liver was known to be oxidative, the oxidation in buffered solution by potassium permanganate was studied. The evidence of kinetic hydrolysis studies, of infrared absorption spectra, of partition coefficients, and of anticholinesterase activity indicated the formation of at least three compounds by oxidation. The most active product had 100,000 times the inhibitory effect of schradan on cholinesterase. It yielded formaldehyde but, on heating or under acid conditions, was converted to a more stable compound which did not yield formaldehyde on hydrolysis. The latter compound was identified as heptamethylpyrophosphoramide by comparison with a synthesized sample. A third compound, of intermediate stability, which did not give formaldehyde, was characterized but not identified (79).

The identity of the most active product of permanganate oxidation of schradan is still under investigation, but it has been shown to be identical to the more active anticholinesterase produced by insects from schradan. This identity has been established by the comparison of hydrolysis constants, of partition coefficients, by infrared spectroscopy and by paper chromatography.

In the search for the reasons for the selective toxicity of schradan to sap-feeding insects, it was found that all insects are able to convert schradan to an active anticholinesterase. Hence the causes of selectivity rest in the site and nature of conversion, and in the fate of the active compounds. The evidence so far obtained suggests that the toxic action of schradan is due only to that fraction which is converted in the nerve cord, and that resistance was conferred by the presence in the insect of extranervous tissue high in converting ability, whereby the schradan becomes activated and hydrolyzed before it can reach the nervous tissue where its intervention would have led to death.

The nature of the enzyme able to convert schradan to an active anticholinesterase was studied in the roach. As schradan is converted by roach gut to an anticholinesterase identical in properties with the more active of the permanganate oxidation products, the enzyme was thought to be an oxidase, though not identical with "trimethylamine oxidase", an enzyme previously described and regarded as involved in the mammalian excretion of choline as trimethylamine oxide. Unfortunately the activity of roach gut is lost on homogenization and could not be recovered by the addition of those co-factors found effective in fortifying liver homogenate. Because it was found that the conversion of schradan is accomplished by many mammalian tissues, of which the most active is the liver, this was used for the study of schradan-converting enzymes. Most of the enzyme activity remained in the supernatant after centrifugation of 10,000 times gravity. The system deteriorated on storage

unless quick frozen and kept in the refrigerator. DPN, magnesium ions and nicotinamide had to be supplied for greatest activity. A further fractionation of the liver supernatant at 45,000 x g. gave a precipitate (microsomes) and a supernatant, which individually were poor converters of schradan but together converted it well. This supernatant acted as a DPN-reducing system, and hydrogen peroxide was detected in preparations actively converting schradan (22, 40).

Incidental to the course of this work on the insecticidal activity of organophosphorus compounds, are observations of possible significance in the correlation of structure and activity. For example, the compound bis[phenyl-di-(2-chloroethyl)phosphoramide]anhydride was intermediate as an anticholinesterase between schradan and monochloroschradan, but was practically non-toxic to the American roach and to the squash bug.

(*c*) *Malathion:*

Malathion has come into widespread insecticidal use since its introduction in 1950 and is of special interest as one of the first organophosphorus compounds to couple high insecticidal potency with low poisonous properties to mammals.

Like other thiophosphates, it is capable of isomerization, and by analogy with parathion, *in vivo* oxidation to "malaoxon" is to be expected. Isomerization was shown to lead to the S-methyl thiolphosphate ("isomalathion") but "malaoxon" has not yet been prepared.

Cholinesterase is inhibited by both malathion and isomalathion, the latter being the more potent. Both produce in insects symptoms of nerve poisoning, but death in the roach is long delayed and does not occur until recovery from cholinesterase inhibition has reached about 57 per cent. Death from typical organophosphorus insecticides usually occurs when cholinesterase activity has been reduced to 10 per cent. The fact that death from malathion does not coincide with the period of greatest inhibition raises the possibility that some other toxic mechanism is at work. However, the figures quoted are based on total cholinesterase activity, and it may be that the pattern of inhibition and recovery at the vital site of malathion poisoning is different from the general pattern.

It was considered important to search for mechanisms other than cholinesterase inhibition. As somewhat similar nervous symptoms may follow a disruption of carbohydrate metabolism, the effects of the insecticide on certain of the important enzymes involved in carbohydrate metabolism were studied. Pyruvate oxidase is interfered with, but only at high insecticide concentrations. Other minor inhibitions were found, but none was adequate to explain the high insecticidal potency and low mammalian toxicity of malathion (48).

(*d*) *Ryania:*

Interest in the ground wood of *Ryania speciosa* Vahl. has been roused by its effective use as a selective insecticide by several laboratories of the Fruit Insect Unit, which requested its study. Lack of knowledge of the insecticidal components of the wood and the non-specific nature of the absorption methods used for the estimation of the alkaloid ryanodine, thought to be its insecticidal component, made bioassay methods necessary, and a search was made for suitable test insects.

Pure ryanodine, a water extract of ryania, and an aqueous solution of the ethanol extract of ryania were used in these tests, and each gave workable probit-mortality regressions on concentration when the test animals were *Drosophila* adults, *Musca domestica* larvae or two species of the water flea *Daphnia*.

Because the alkaline hydrolysis of ryania yields pyrrole-2-carboxylic acid, the ultraviolet spectrum of which is closely related to that of ryanodine, tests were also made of this compound and its ethyl ester. The ethyl ester was toxic to *Daphnia*.

(*e*) *Bioassay of insecticide residues:*

A method for estimating directly insecticide residues in plant materials has been developed. It is based on the mortality of adults of *Drosophila melanogaster* Mg. exposed on smears of the macerated plant material. The sensitivity and reliability of the method were shown to depend on the age, sex ratio, and handling of the flies, and on the technique of exposure and replication of the tests. Statistical methods were adapted to test the significance of the estimates. Residues of 0·05 ppm. of aldrin, for instance, could be estimated (42).

(*f*) *Insect Rearing:*

The laboratory is equipped with six insect-rearing rooms, in each of which temperature and humidity can be controlled to meet the requirements of different insect species. The six rooms are arranged as a block provided with an independent air-circulation system and separated by two air locks from other parts of the building.

Rearing methods are in the main standard, but the following precautions and modifications of published methods may be noted. The fruit fly *Drosophila melanogaster* is reared continuously on a custard pumpkin medium to which a stiffening matrix of cellucotton is added to permit the larvae to penetrate deeply enough to complete feeding. The vigor, viability and fecundity are thereby improved, the production of adults per egg-laying female rising from 8 to 30, and the average fly weight from 0·6 to 1·0 mg.

The cadelle *Tenebroides mauritanicus* L. is of special interest, for it is resistant to halogenated hydrocarbon fumigants such as methyl bromide and ethylene dibromide. This insect had previously been found difficult to rear in quantities large enough for test purposes until it was observed that the female prefers to oviposit in small crevices. A site for oviposition was therefore provided by placing two squares of transparent plastic, one above the other, and separated by a spacer of thin paper. The eggs are laid in clusters between the plastic plates and can be seen and counted easily. The eggs are transferred daily to culture bottles containing whole wheat flour to which is added a small amount of brewer's yeast. For pupation, a small cube of coarse insulating cork is provided. By this method up to 10,000 mature larvae are produced each week. This method is now widely used elsewhere for not only is the cadelle of high economic significance in ship fumigation but the larvae are excellent test organisms for the examination of the resistance of food packages to insect penetration (28).

Rearing the housefly, *Musca domestica* L., is continuous and differs from the Standard C.S.M.A. method used in Canada and the United States. Larvae are reared in battery jars on minced cellucotton impregnated with evaporated whole milk and dried brewer's yeast. Constant effort is being made to improve the size, vigor and uniformity of the flies so that they may be used with confidence in precise physiological and toxicological tests. The method has several distinct advantages over the alfalfa-bran-yeast larval medium: the milk medium is relatively odorless, pupae are screened from the sawdust on the medium in a very few minutes without prior drying; the temperature of the medium can be controlled; and the production can be varied easily to suit the need.

Among other insects reared by routine methods are: the milkweed bug—*Oncopeltus fasciatus* Dall., the pea aphid—*Macrosiphum pisi* Kalt., the leaf

hopper—*Macrosteles divisus* Uhl., the Mediterranean flour moth—*Ephestia kuehniella* Zell., the Oriental fruit moth—*Grapholitha molesta* Busck., the roach—*Periplaneta americana,* the squash bug—*Anasa tristis* Deg., the granary weevil—*Sitophilus granarius* L., the flour beetle—*Tribolium confusum* Duv., and the mosquito—*Aedes ægypti* L.

HERBICIDES

The Metabolism of Indoleacetic Acid

The effects of many herbicides such as 2,4-D and maleic hydrazide on plants strongly suggest that their action is a disruption of the normal hormonal control of plant growth. Although the morphological effects of the natural auxins, of which indoleacetic acid is the only member known with certainty, have long been recognized, little is known of the metabolic and biochemical means by which indoleacetic acid affects growth. An enzyme system, indoleacetic acid oxidase, is known to be present in many plant extracts and this system is capable of a rapid *in vitro* destruction of indoleacetic acid, though it is not known whether it is operative in intact plants. Nor have *in vitro* studies given a comprehensive picture of its function in growth regulation. Hence, before studying the effect of herbicides on auxin metabolism, it was necessary to examine auxin metabolism itself, for which purpose two approaches were used: first, the *in vitro* decomposition of indoleacetic acid both biochemically and photochemically; second, the uptake and metabolism of indoleacetic acid by intact plant tissues.

One route by which indoleacetic acid is decomposed *in vitro* involves an oxidase system, which probably contains at least two enzymes, a flavoprotein and a peroxidase. It was thought that the flavoprotein oxidizes indoleacetic acid with liberation of hydrogen peroxide which is then utilized by a peroxidative reaction. The production of hydrogen peroxide was demonstrated by a fluorometric method involving scopoletin. Scopoletin is a peroxidase substrate, and the extent of its oxidation is directly proportional to the peroxide concentration of the reaction mixture within the range 1 to 20 x 10^{-6} moles of hydrogen peroxide. This property has been utilized in a highly sensitive and specific method for the estimation of hydrogen peroxide in biological materials (44).

The rate of oxidation of indoleacetic acid by the enzymic process is not affected by the concentration of endogenous hydrogen peroxide as was reported by earlier workers. The reaction is stimulated by maleic hydrazide, by methyl umbelliferone and by 2,4-D. Although it is possible that the increased rate of destruction of indoleacetic acid thus brought about by some of these weed killers may be a cause of their growth-inhibitory action, this explanation cannot be applied to 2,4-D. This compound is a powerful herbicide at concentrations much lower than those required for an appreciable effect in the *in vitro* oxidation of indoleacetic acid (15).

The contrasted effects of methyl umbelliferone and scopoletin, both related naturally-occurring fluorescent coumarins, is of interest. Methyl umbelliferone, which (as mentioned above) accelerates indoleacetic acid oxidation, is unaffected by peroxidase; scopoletin, which is oxidized by peroxide, is a competitive inhibitor of indoleacetic acid oxidase. At low concentrations, scopoletin therefore slows down the rate of indoleacetic acid oxidation, and this may be the mechanism by which the compound stimulates growth (5).

This difference in action between scopoletin and methyl umbelliferone operates only in darkness, for in the light a non-enzymic process comes into play. Both of the coumarin derivatives accelerate this photo-induced oxidation

of indoleacetic acid, just as both derivatives were found to catalyse the photo-induced oxidation of manganous ions by riboflavin in the presence of catalase and pyrophosphates. Both of these photo-reactions are, however, inhibited by a third fluorescent coumarin derivative, esculetin. A possible explanation of this difference rests in the ability of monohydric phenols to catalyse both photooxidations, whereas the quinone-forming dihydric phenols inhibit these reactions. Both scopoletin and methyl umbelliferone have one hydroxyl group: esculetin is 6,7-dihydroxycoumarin (46).

Maleic hydrazide was also found to accelerate the photooxidation of manganous ions and therefore simulates a monophenol. For this reason, a polarographic study of maleic hydrazide and its methyl derivatives was undertaken; the results indicated that maleic hydrazide reacts as if it were a monobasic acid of pK_a 5·65, bringing it into line with other monohydric catalysts of the photooxidation reaction (57).

The uptake of indoleacetic acid by intact tissue was examined by placing stems in solutions of indoleacetic acid of concentrations not far removed from those exerting the greatest stimulus to cell elongation. Under these conditions pea stems readily removed indoleacetic acid from the solution, although none could be indicated in the plant tissue itself. At higher concentrations indoleacetic acid appears in the plant tissue in amounts proportional to those of the solution. In both cases, however, substances appear in the plant tissue which give a mauve color with Salkowski's reagent, which with indoleacetic acid gives a pink color. In pea tissue the mauve Salkowski reaction has been traced to indoleacetylaspartic acid.

From tests in which tissue of plants other than pea were used, it was found that the formation of indoleacetylaspartic acid is typical of legumes, but that in grasses the applied indoleacetic acid is chiefly conjugated with ammonia to give indoleacetamide. A number of other Salkowski-reactive derivatives have been detected, but usually in small amounts. A substance still unidentified, which gives a pink color with the Salkowski reagent, is destroyed on hydrolysis without liberation of indoleacetic acid, and is presumably an intermediate of the metabolism of indoleacetic acid.

Indoleacetylaspartic acid has no effect on the growth of peas but is active on monocotyledons, presumably through a regeneration of indoleacetic acid. Its formation may represent a detoxication process maintaining indoleacetic acid at a low level in the pea tissue, but it is not known whether this reaction occurs with endogenous indoleacetic acid. Nor is the fate known of that part of the indoleacetic acid which disappears from the solution in which the plant tissue is placed, for only about one-fifth of it is recovered as indoleacetic acid or indoleacetylaspartic acid from the pea tissue. Preliminary evidence suggests that the missing amount is oxidized to products which no longer give a color with the Salkowski reagent (49, 62 and 64).

2,4-D:

The annual consumption in Canada of 2,4-dichlorophenoxyacetic acid, its salts and esters quickly reached the 3,000,000 lb. mark, and though this wide use has not revealed any serious deleterious or beneficial effect on the soil micro-organisms, it is necessary to know the fate of this material in the soil. The ability of certain soil micro-organisms to decompose 2,4-D has been shown, but little is known about their physiology.

A first requirement being methods for the detection and estimation, in soil and culture media, of 2,4-D and its likely breakdown products, a suitable quantitative colorimetric method for 2,4-D was devised together with chromatographic methods for the resolution and detection of some phenols.

By suitable enrichment techniques a small, gram-negative, non-sporeforming, ærobic, motile, rod-like bacterium able to metabolize 2,4-D was isolated from humus-rich soil. Its taxonomic position is not clear. Studies of its nutrient requirements in agar culture containing 2,4-D show that the organism grows especially well with the addition of the dicarboxylic acids of the Krebs cycle, bicarbonate, formate, urea and histidine. In the absence of 2,4-D the organism grows well in the presence of the common pentoses and shows a high specificity for 2,4-D as growth substrate, but it can also utilize 2-methyl-4-chlorophenoxyacetic acid. The metal and phosphate contaminants of the nutrient media are strong enough to meet requirements for growth, and only the addition of strong chelating agents reveals the necessity of some metals. In liquid media the organism requires added calcium or magnesium for optimum growth. Growth on common bacteriological media is poor or fails rapidly, but the organism has now been grown for over a year with many transfers on a new synthetic medium without the loss of vigor noted by other workers for 2,4-D-decomposing bacteria.

The responses of the organism to 2,4-dichloro-6-hydroxyphenoxyacetic acid, 2,4-dichlorophenol and 3,5-dichlorocatechol, which are theoretically possible degradation products of 2,4-D, were examined using intact cells. All of these compounds were metabolized by the cells, but none in the manner expected of an intermediate. For example, an initial lag in the oxygen-consumption curve occurs, which would not be expected if the compound were an intermediate. Moreover, the ultra-violet absorptive spectra of the metabolized compounds differed from the spectra of the metabolized products of 2,4-D. The high molar ratio of oxygen to 2,4-D metabolized in the Warburg respirometer, and the fact that neither phenolic nor carbonyl groups could be detected by the *p*-nitrosodimethylaniline and the 2,4-dinitrophenyl hydrazine reagents, suggest that 2,4-D is broken down to small units.

Although 2,4-dichlorophenol is more vigorously oxidized by the organism than is 2,4-D, the phenol is highly toxic to growing cells. The addition of 0·1 per cent yeast to a liquid medium containing 2,4-D causes the cells to accumulate 2,4-dichlorophenol, with the subsequent death of the organism. The "blocking agent" which prevents the metabolism of the phenol has not been identified but it is ether-soluble, easily adsorbed on charcoal, stable to autoclaving at pH 7 and is not thioctic acid nor a common amino acid.

Complementary to the study of bacterial decomposition of 2,4-D, its photochemical degradation has been examined. The irradiation of neutral 0·1 solutions of 2,4-D with ultraviolet light yields at least four phenolic compounds. Irradiation with visible light in the presence of riboflavin gave 2,4-dichlorophenol, but under these conditions no phenol was detected when any one of 2,4-dichloroanisole, sodium 2-(2,4-dichlorophenoxy)ethyl sulphate, 2-(2,4-dichlorophenoxy)ethanol or 3-(2,4-dichlorophenoxy)propionic acid was used instead of 2,4-D. These results are evidence that the rupture of the molecule at the ether bridge is aided by the electronegative carbonyl group of 2,4-D, an effect shown only when the ethereal oxygen is linked to the α-carbon (63).

CMU:

CMU (3-(4-chlorophenyl)-1:1-dimethylurea) has rapidly come into use as a soil sterilant and pre-emergent herbicide, but its mode of action is unknown. Although applied directly to the soil, applications of 10 lb. per acre or less of CMU have little, if any, effect on the germinating seed or on the radicals of young plants. The seedlings produce tops before the toxic action of CMU becomes evident. Plants of kidney bean or of corn have been killed with applications of as low as 2 ppm. CMU but, when the seedlings were

grown in a graded series of concentrations of CMU in water, more than 32 ppm. were required to give a suppression of root growth. Furthermore when potted tomato plants were watered with 32 ppm. CMU, the roots remained active for some days after the leaves had been killed. These observations lead to the conclusion that the primary site of action of CMU is in the foliage and shoots (69).

Studies were made of the routes whereby CMU reaches the leaves and the shoots. Roots of intact tomato and kidney bean plants were placed in CMU solutions. Symptoms in the leaves appeared almost as fast as when tops with cut stems are placed in other solutions of the same CMU concentrations. Hence CMU is readily accessible to the vascular system of the intact plant. Moreover if one of the pair of primary leaves of bean is shaded, the appearance of symptoms is delayed in that leaf. Hence CMU moves in the transpiration stream and accumulates in the leaf tissue as the water is transpired. Mature leaves are affected before young expanding leaves because the CMU accumulates more rapidly in the leaves with higher rates of transpiration. Likewise necrotic blotches in individual leaves, because of differences in stomatal frequency, develop first at the margins and tips of leaves (33).

In symptomological studies, acute and chronic effects were distinguished. The acute effects, primarily a water-soak necrotic blotch, appear at internal concentrations of about 100 micrograms per gram of fresh leaf and develop in a few hours under conditions conducive to rapid transpiration, and at external concentration of 128 ppm. CMU. Chronic effects, including wilt, chlorosis, stem collapse of corn and silver blotch of bean, are produced at internal concentrations of less than 50 micrograms per gram fresh leaf and by external concentrations of one to two ppm.; they require several days to develop. Chronic symptoms would predominate from pre-emergent applications of four lb. or less CMU per acre.

In a search of the explanation for the difference between water-soak blotch and silver blotch of bean leaves, a microscopic examination showed that, in water-soak blotch, both the palisade and spongy parenchyma were disrupted. The destruction of cellular membranes, and the consequent leakage of cell contents, produces the water-soaked appearance of the tissue, which remains green because the disorganization of chloroplasts comes later. In silver blotch the spongy parenchyma stays normal but the palisade parenchyma is destroyed, permitting the entry of air which produces the silver appearance of the upper leaf surface.

Light intensity has been found to exert an influence on the rate of symptom development to an extent greater than could be explained solely through its effect on transpiration. This effect is sufficiently strong to suggest that the diversity of results reported in field trials of CMU may be due, in part at least, to the effect of exposure site, degree of shading or season, on the phytotoxic response to CMU. Treatment of excised bean leaves with CMU reduces the production of dry matter to an extent that indicates that this chemical may be acting as a photosynthetic poison (73).

FUMIGATION

In recent years fumigants have been used in Canada to solve a number of problems, some of which have been of a regulatory nature. Examples of this type of work are the fumigation of large quantities of imported plant products or the disinfestation of ocean-going ships before they are loaded with grain. An expanding use of fumigants is their large-scale application to the soil. While the early use of fumigants was largely concerned with insect control, several gases have found use against pathogenic micro-organisms.

In some fields of use, such as soil fumigation, the desirable aim of comprehensive treatments to control insects, fungi, bacteria and eelworms seems already to have been achieved.

Fumigation research is one aspect of applied biology in which laboratory work may be closely linked with practical application. An investigation is constantly enriched if the personnel engaged in it are able to work in, or can keep in close touch with, both settings. Hence, close contact is kept between both aspects of the work. The work of the fumigation section is based on the need for laboratory experimentation under closely controlled conditions, supplemented when necessary by work in the field.

Fumigation Equipment

The first step was the installation of suitable equipment. The basic idea in its design has been to provide the means of establishing and maintaining, for short or long periods, controlled conditions of temperature and humidity. The principal feature of the equipment is the battery of seven fumigation chambers which are housed in an insulated room. The fumigants introduced into the chambers are discharged into the outside air at the end of the treatment by means of a pump. In case of emergency the room itself may be ærated by springing a trap door in the ceiling leading to an exhaust duct and blower. The temperature of the room may be established over a range of —7°C. to 35°C. with a maximum variation of ±1°C. by means of an electrical-pneumatic controller of conventional design. The controller records and is capable of direct setting to any temperature within the operating range of the system. Transfer of heat through the steel wall of the fumigation chambers permits a rapid equalization of temperature within and outside the chambers, and temperature control is maintained satisfactorily when the chambers are evacuated to the lowest possible pressure of 8 mm. Hg. attainable with the vacuum pump employed. At temperatures between 20° and 30°C. the humidity within the room can be accurately controlled between relative humidities of 50 and 100 per cent. It is also possible to maintain close control of the humidity within the chambers if the total volume of insects or organisms is small compared with that of the chamber. If the doors of the chamber are left open before the fumigation begins and the humidity brought into equilibrium with that of the room, the relative humidity so obtained will persist inside, after the doors are closed, for periods of at least 24 hours.

Six of the fumigation chambers within the room are of similar dimensions while the seventh is a larger unit for accommodating nursery stock or trees. The chambers have walls 3/16 inch thick and the doors are tightly closed by a conventional clamping method. The system of pipes connecting the chambers with the exhaust pump is so arranged that recirculation of the fumigant-air mixture in two of the chambers may be effected if desired.

An original feature of the six regular test chambers is the so-called dispenser. This is a cylindrical steel vessel mounted on the main chamber, with walls of the same thickness. By means of a movable port with gasket, the dispenser may be sealed off from the main chamber and evacuated separately. This dispensing mechanism is designed for many types of manipulation:—(1) For completely evaporating known weights of liquids before the vapors are brought into contact with test organisms or commodities, so that the timing of the fumigation may be accurately set. (2) For accurately measuring, by means of a gauge or manometer, the volume of a gas or mixture of gases introduced into the dispenser. (3) For homogenizing mixtures of fumigants before they are introduced to the test material. (4) For conveniently studying phenomena, such as that referred to as protective stupefaction, by closing the dispenser and evacuating it at any time after the beginning of the

experiment without disturbing the test system, when another gas, or even a sequence of gases, may be introduced. (5) For progressively increasing or decreasing the concentration of a gas in the main chamber without altering the pressure or other conditions in the test system.

There are six threaded 1″ pipe inlets in the walls of the main chambers. Copper tubes running through pipe plugs threaded in the inlets may be used for withdrawing samples of the gas-air mixture from different parts of the fumigation system as desired.

The chambers may be equipped for studying the effect of concentration on given organisms over different periods of time. Narrow troughs may be inserted through the threaded inlets described above so that individual insect cages in a train can be withdrawn or inserted at predetermined intervals of time. The cages have tight-fitting flanges at either end which prevent significant loss of fumigant as each cage is moved in or out. A train of these cages may be seen in the open chamber at the left of Figure 3.

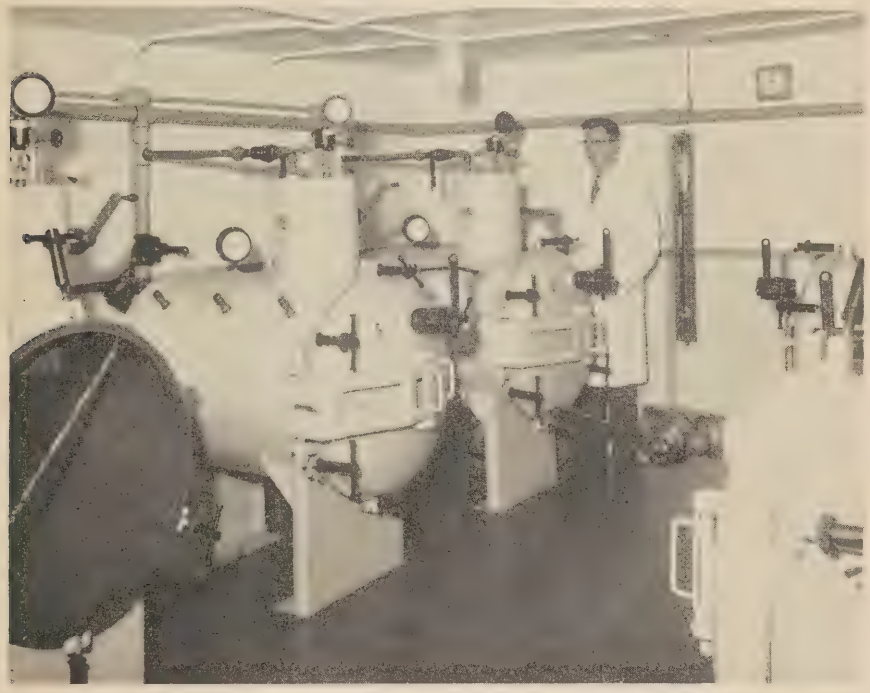

Figure 3—A corner of the Fumigation Room showing fumigation chambers, one with door open.

In order to avoid a heating effect and to reduce hazards, the motors driving the fans are mounted on the outside of the chambers and the shafts are led through vacuum seal units.

Effect of Fumigants on Insect Populations

There are certain types of vacuum fumigation in which complete mortality of the offending organisms can be obtained. However there are many fumigation treatments at atmospheric pressure in which something short of 100 per cent kill may be expected. This is especially true in the treatment of buildings and transportation carriers, or in field treatments conducted under movable gas-proof sheets (tarpaulins). There is not only the problem of the emergence of resistant strains as the result of such a program, but also the question of

delayed effects of the treatment on survivors, such as the stimulation or retardation of oviposition, alteration in the biotic potential of the population or abnormal growth and development of progeny. The study of these effects is an integral part of the toxicology of fumigation.

(*i*) *Toxicity of fumigants to insects:*

For a proper understanding of the responses to fumigants of the individual insect or populations of it, it is necessary to have accurate data on the intrinsic toxicity of the fumigant being studied. The cadelle *Tenebroides mauritanicus* has been selected as one of the principal insects for study, because it is more resistant than *Sitophilus granarius* or *Tribolium confusum* to the following commonly used fumigants: acrylonitrile, methyl bromide, ethylene dibromide, ethylene chlorobromide, ethylene dichloride, carbon tetrachloride, ethylene oxide and sulphur dioxide. An interesting exception is that chloropicrin is more toxic to the cadelle than to the other two insects.

(*ii*) *The development of resistant strains:*

One of the earliest records of the development of resistance to insecticides was the reaction of the California red scale to hydrocyanic acid gas. In recent years the halogenated hydrocarbon fumigants have become extensively used against stored product insects, but there has been no report of a development of resistance. In view of the practical importance of this problem, populations of the three species of stored product insects mentioned above have been subjected to the selective action of methyl bromide since early in 1953. At the end of 1955 two stocks of *S. granarius* selected from 11 and 14 generations respectively had reached a degree of tolerance such that the dosage required to kill the population at any mortality level was twice that required for the original unselected stock (61).

(*iii*) *Effect of fumigants on biotic potential:*

Apart from the problem of development of increased tolerance, there is the general question of the ability of survivors to re-establish populations. Such a question involves a study of a number of biological factors such as resultant fertility of survivors, the viability of the eggs laid and the fertility of the next and following generations. In a preliminary investigation it was observed that, following fumigation of adult females of *T. mauritanicus*, some lived for more than three months without laying eggs, while others resumed normal frequency and amount of oviposition after a delay of about two weeks.

(*iv*) *Effect of fumigants on insect respiration:*

As part of the study of the mode of action of a fumigant it is desirable to know the respiration response during exposure to the fumigant. Perhaps technical difficulties discouraged earlier workers from attempting this investigation, but a method has been developed for determining the rate of oxygen consumption of insects during fumigation. The conventional Warburg respirometer is used allowing a known concentration of the fumigant to enter the flasks, each of which contains one or more of the insects being studied. The fumigant-air mixture is previously made up in a large chamber and the fumigant concentration determined by analysis. The mixture is then pumped into the individual flasks of the respirometer, and oxygen consumption determined in the usual manner.

So far methyl bromide has been the fumigant used, principally in a study of the responses of the fourth-instar larva and adult of *T. mauritanicus*. The response of these insects during a five-hour fumigation at 25°C. was determined at three dosage levels; one causing 99 per cent mortality; one giving 50 per cent mortality; and one at the maximum sublethal dose. The latter level is an amount which, while allowing complete survival, still constitutes an

appreciable dosage of the fumigant. A study of the effects at this low level is necessary because some species of insects enter a stupefied state when exposed to a sublethal concentration, and thereby protect themselves from subsequent treatments.

In the response of the cadelle larvae to methyl bromide, there is no apparent effect on respiration at any time during exposure at the three dosage levels. However, there is a significant correlation between the intrinsic respiratory rate of the individual larvae and their susceptibility to the fumigant. The average oxygen consumption of the victims was 30 per cent higher than that of the survivors. There was no decrease in respiratory rate during exposure to sublethal doses, indicating that no protective effect was initiated. Treatment of the insects with a sublethal dose prior to a lethal dose showed that no protection was afforded by this initial exposure (78).

Theory and Practice of Vacuum Fumigation

Vacuum fumigation to control insects and other organisms is a technique which is widely employed especially in food industries and plant quarantine work. In a field study of the penetration of methyl bromide during the vacuum fumigation of jute bags, it was found that the region of insecticidal weakness in this treatment might lie in the free space of the chamber at the periphery of the highly compressed bales, rather than within the bales themselves (29).

The laboratory examination of this unexpected effect was therefore undertaken. Jute bags compressed into bales were chosen for this work, since jute in this form is particularly suitable for several reasons; it is inert and the same material can be used repeatedly if ærated between tests; it is highly sorptive and thus it provides the opportunity of studying the critical subject of fumigant sorption; it is hygroscopic and by suitable manipulation of surrounding humidities the bales can be adjusted for observations on the effect of varying moisture content. Because methyl bromide is, at present, commonly used for the disinfestation of jute it was chosen as a fumigant for the general study.

Two methods were used to determine the distribution of the fumigant: the chemical analysis of small samples removed at intervals of time from different parts of the system; the assessment of the effect on test insects placed at predetermined positions within and around the bales.

The various manipulations of the pressure of the system were: (*a*) varying the initial vacuum drawn on the system before the introduction of the fumigant; (*b*) sustaining the pressure of the system after the fumigant is applied (sustained-vacuum fumigation); (*c*) restoring to atmospheric pressure, either gradually or quickly at different times subsequent to the introduction of the fumigant, by allowing air to flow into the chamber ("atmospheric pressure restored" fumigation, formerly known as "vacuum dissipated" method); (*d*) the simultaneous introduction of the fumigant, already in the gaseous condition, with the air stream so that a homogenous mixture of air and fumigant flows in until atmospheric pressure is restored.

In all combinations with the temperature remaining constant at 25°C. the most important modifying factor is the moisture content of the jute. At moisture contents of above 14 per cent (dry weight basis) but below complete saturation, the fumigant diffuses rapidly throughout the system, and at a given dosage it is difficult to demonstrate the advantage of one method over another. When moisture is low, significant differences can be demonstrated between different types of treatment. Also when the vacuum is sustained for any length of time with normal (9-10%) or low (7%) moisture content, during the treatments (*a*) and (*b*) above, there is an optimum working

pressure at approximately 100 mm. Hg. Because the treatment recommended has to be that effective at the lowest conceivable moisture content, comparisons between methods have been made at the 7 per cent moisture level. The most promising methods for three hours exposures so far tested are listed in descending order of efficiency:—

(1) An initial pressure of 18 mm. Hg. is produced in the system and, after introduction of the fumigant, the chamber is slowly brought back to atmospheric pressure, the optimum time for the restoration process being 90-120 minutes. The pressure is held at atmospheric to complete the three-hour exposure. This could be described as a method of "slow restoration of atmospheric pressure from initial low pressure".

(2) An initial pressure of approximately 90 mm. Hg. is adjusted to 100 mm. by the introduction of the fumigant. The pressure is held at this level for 42·5 minutes. Then atmospheric pressure is restored rapidly in 2·5 minutes and held there for 135 minutes, the remainder of the three-hour exposure. This constitutes essentially the "atmospheric pressure restored" method of Burns, Brown and Heuser (*J. Sci. Food Agr.*, 1953, *1*, 48 and 378).

(3) After an initial pressure of 90 mm. Hg. is produced, the fumigant is introduced and the pressure held at 100 mm. Hg. for the entire exposure period of three hours. This is the classical method of sustained vacuum fumigation.

(4) A vacuum at 42 mm. Hg. is adjusted to 50 mm. by the introduction of the fumigant. Atmospheric pressure is restored immediately (2·5 minutes) and the exposure completed at this pressure. This is the method of "immediate restoration", and it was found to effect a poor distribution of fumigant which results in a marked variation in insect mortality throughout the system.

(5) After evacuation to an initial pressure of 18 mm. Hg., the fumigant-air mixture, previously thoroughly homogenized, is allowed to flow quickly into the system until atmospheric pressure is reached. This method results in poor penetration of fumigant to the center of the bale and low mortalities within the bale. This is the method of "simultaneous introduction" of Lepigre (*Document phytosanitaire No. 9, Min. Agric. France,* Algiers).

These findings are supported by chemical analysis, which is used to derive concentration (c) $\times$ time (t) products for the various positions. It was found that ct products are correlated with insect mortality.

Fumigation concentrations in structures and their relation to insect toxicity:

Fumigants can be used in structures not specifically designed for fumigation, but in which it is convenient to deal with infestation problems. Such structures include any buildings which can be rendered reasonably airtight, such as houses, mills, grain elevators and warehouses, and transportation carriers such as ships, barges, railway freight cars and trucks. The double purpose is served of disinfecting the material and destroying the insect populations in the structure, so that goods subsequently loaded or carried are not contaminated. This type of treatment, employing methyl bromide as the fumigant, is widely used in Canada. The dosage schedules recommended were based on laboratory observations on the toxicity of this fumigant to the insects being controlled, backed by field observations on the success of the treatments; but in none of this work was the opportunity afforded to determine, by chemical or other means, the actual concentrations present throughout the structures during the exposure period. Nor was there any reliable information on the degree of loss of fumigant by leakage from these structures.

Since the inception of the work at this laboratory, opportunities have been afforded to remedy some of these deficiencies. This work has been aided by two developments. Firstly, the equipment in this laboratory has enabled

accurate studies to be made of the reaction between the fumigant and the actual commercial package or bag containing the material fumigated in the structures. Secondly, the application of the principle of gas analysis by thermal conductivity to the determination of methyl bromide provides the needed analytical method.

The study of the gas concentrations in an empty cargo ship demonstrated that methyl bromide, applied from near the top of the hold and distributed by means of conventional ¼ h.p. fan at the bottom, was evenly distributed soon after the beginning of the treatment. A high level of concentration of 81 per cent of the nominal dosage was recorded at the end of the 10 hour exposure period. After aeration was started, by removal of tarpaulins and some of the hatch covers, in the usual way, all the detectable concentrations of gas had disappeared within 90 minutes (27).

The behavior of methyl bromide during the fumigation of shelled peanuts in a lake freighter and a railway box car was also studied. It was shown that leakage in either of these structures was not an important factor and that the progressive fall in the nominal concentration of the fumigant could be attributed to the degree of sorption expected from parallel treatments in the laboratory chambers. The *ct* products calculated from the observations indicated that all common stored product insects, including those present in the shipments, would be completely controlled with one possible exception. The *ct* product in the railway car was slightly below that desired from laboratory observations for 99·9 per cent mortality of the cadelle *T. mauritanicus* (67).

ECOLOGICAL CHEMISTRY

Toxin Production by *Helminthosporium sativum*

Helminthosporium sativum B.K. & B. is responsible for a seedling blight, foot- and root-rot, head blight and leaf spot of cereals and grasses. The induction of the seedling blight phase of the disease on barley is associated with the production, by the fungus, of toxic substances which are not only prerequisite for the invasion of the host tissue by the pathogen but which may predispose the roots and basal parts of the plant to attack by other micro-organisms not normally regarded as pathogens*.

The isolation and identification of these toxic substances involves an initial study of their production by the fungus when grown in artificial culture which, in turn, requires methods for their evaluation. The powerful inhibition of germination of barley seed by the toxins was at first used to detect their presence and to assess the potency of the extract. In later work, this method of bioassay has been supplemented by the standard fungicide tests using spores of *Sclerotinia fructicola*, for the toxins are strongly anti-fungal. A complete correlation between the two bioassay methods has thus far been obtained.

H. sativum is able, contrary to previous experience, to grow very rapidly in artificial culture when its growth requirements are adequately met. Given good aeration, growth was excellent on a variety of media, though slower on media wholly inorganic except for the addition of sucrose or dextrose as energy source. The best production of toxins, however, has been obtained when an organic source of nitrogen is used. Peptone, used in initial experiments, was found to create difficulities not only because it was able itself to inhibit to some degree the germination of barley, but also because it gave degradation products which complicated the subsequent extraction of toxins. Replacement

*For further detail see: Ludwig, R. A., R. V. Clark, J. B. Julian, and D. B. Robinson. "Studies on the seedling disease of barley caused by *Helminthosporium sativum*, P.K. & B." *Can. J. Bot.* (in press).

of peptone by one or more of a range of amino acids was not satisfactory, and finally potato-dextrose (or potato-sucrose) liquid medium was adopted for use in the 100 liter fermentor employed in isolation studies.

Maximum toxin production coincides with the onset of autolysis, and growth is frequently self-limited through toxin production. An increase in the concentration of the medium increases growth but not toxin production. In a concentrated medium apparent activity reaches a maximum and then decreases rapidly, whereas in a dilute medium it is sustained for a longer period and extraction time becomes less critical.

The activity of culture filtrates is retained over a wide pH range. Some loss occurs on heating at 60°C. for 16 hours at pH 10·0 but none at pH 4·0. On concentration in a vacuum flash evaporator activity is divided between the residue and distillate. Extraction of the still residue with chloroform, after a preliminary alcohol precipitation of inert material, removes the activity and yields a product which completely inhibits the germination of *Sclerotinia* spores at 100 ppm. and the germination of barley seed at 200 ppm. Alkali treatment of the chloroform extract indicates the presence of neutral and acidic fractions.

The activity found in the distillate can be removed either by direct ether extraction or by adsorption on charcoal and elution with acetone. The product thus obtained is at least equal in activity to that found in the residue. Infrared absorption spectra of the two products are very similar. The presence of terminal methylene groups is indicated by both infrared absorption and ozonolysis. Both products have ultraviolet absorption peaks at 262-265 mμ.

Bacterial Dissociation

The inconsistency of many fungi and bacteria when grown in artificial culture is notorious and, in a number of instances, the changes in character are induced by environmental factors. At one extreme is the bacterial transformation reaction which has been defined "as a hereditary alteration in a susceptible cell resulting from the acquisition from its environment, by other than sexual means, of a genetically active unit directing the inheritable change". At the other extreme is the selection, by the continued use of an antibiotic such as penicillin, of pathogenic bacteria possessing extreme tolerance to this bactericide. An intermediate phenomenon is the dissociation prevalent among bacteria in which one form, such as the strain of *Rhizobium* effective in establishing symbiosis with the appropriate legume and of fixing atmospheric nitrogen, is converted by change of environment or nutrient to another strain ineffective in nitrogen fixation, or to one wholly parasitic in its attack on its host legume. The application to the soil of a chemical of high biological activity such as an insecticide or weed killer may perhaps result in an unwanted and deleterious dissociation not only of *Rhizobium* but of other microorganisms of agricultural significance.

Clearly, a closer study of this possibility in the case of *Rhizobium* requires methods for the growth of the legume under sterile conditions. Three attempts have been made to grow clover under such conditions in the greenhouse, but in every case a spell of hot weather ruined the plants. It was therefore decided to await the construction of plant growth rooms before pursuing this work further, and meanwhile a study was begun of the ubiquitous soil bacteria *Bacillus cereus* Frankl. & Frankl. *B. cereus* has several variants, some of

which are pathogenic to insects. The examination of the sporulation of certain of these variants revealed parasporal bodies, of which that found in *B. thuringiensis* Berliner, has so far received the most attention. Under the light microscope the parasporal body is revealed as a highly refractile and well-formed diamond-shaped crystal. Each sporulated cell contains one spore and at least (and usually only) one crystal. On completion of sporulation the spore remains within the exosporium, but most of the crystals are free. Cultures of other variants of *B. cereus* pathogenic to insects were examined, and all were found to form crystals on sporulation. Strains of non-pathogenic *B. cereus* collected from soil and foodstuffs failed to form crystals on sporulation (24).

These findings prompt the speculation that crystal formation is a genetical characteristic of the organism which is, in some way, connected with the formation of a toxic substance causing septicaemia in insect larvae. This speculation attracted the attention of Dr. T. E. Angus, of the Insect Pathology Laboratory at Sault Ste. Marie, then working on *B. sotto* Ishiwata, which is pathogenic to silkworm larvae. Using a method devised here for the separation of the crystal material from the spore, he was able to feed the silkworm with the crystal material. At a dosage rate of 0·5-1·0 μg. per gram larvae paralysis resulted within six hours; the spore had no effect. To provide an idea of the extreme potency of the crystal protein, it may be mentioned that calculation reveals that it is about 200 times as insecticidal to silkworm as the toxin derived from *Corynebacterium diptheræ* (Flügge) Lehmann & Naumann.

The possibility remains that the toxicity of the crystalline material was associated with virus or phage. Electron microscope studies have revealed no evidence of virus particles even when the crystal is dispersed by treatment with alkali. Moreover the examination of ultrathin sections of the bacterium at this stage of the sporulation process has provided electromicrographs that reveal no evidence of the presence of virus or of phage infection. One such photograph is represented in Figure 4.

Because of the insecticidal properties of the parasporal body it has been subjected to chemical examination. The purest preparation of the crystal material yielded a substance precipitated by trichloroacetic acid possessing the ultraviolet absorption characteristic of a protein, it contained no phosphorus, over 17 per cent nitrogen and at least 17 amino acids, all α-amino acids commonly found in proteins (55).

The toxicology of this bacterial protein poses a fascinating problem. The death of the affected larvae is due to a general septicaemia which follows paralysis. It would be of interest to know whether there is a dosage of the protein material which, without killing the larvae, would permit the infection of the tissue by the bacterial flora of the gut, by those pathogenic strains of *B. cereus* which form inclusion bodies, or by related non-infective strains of *B. cereus* such as *B. mycoides* and the avirulent *B. anthracis* (74).

Meanwhile the study of the phenomenon of dissociation in *B. cereus* is continuing. The change of the growth habit, which is the basis of the differentiation between *B. cereus* and *B. thuringiensis,* has been frequently obtained, but the underlying cause remains elusive.

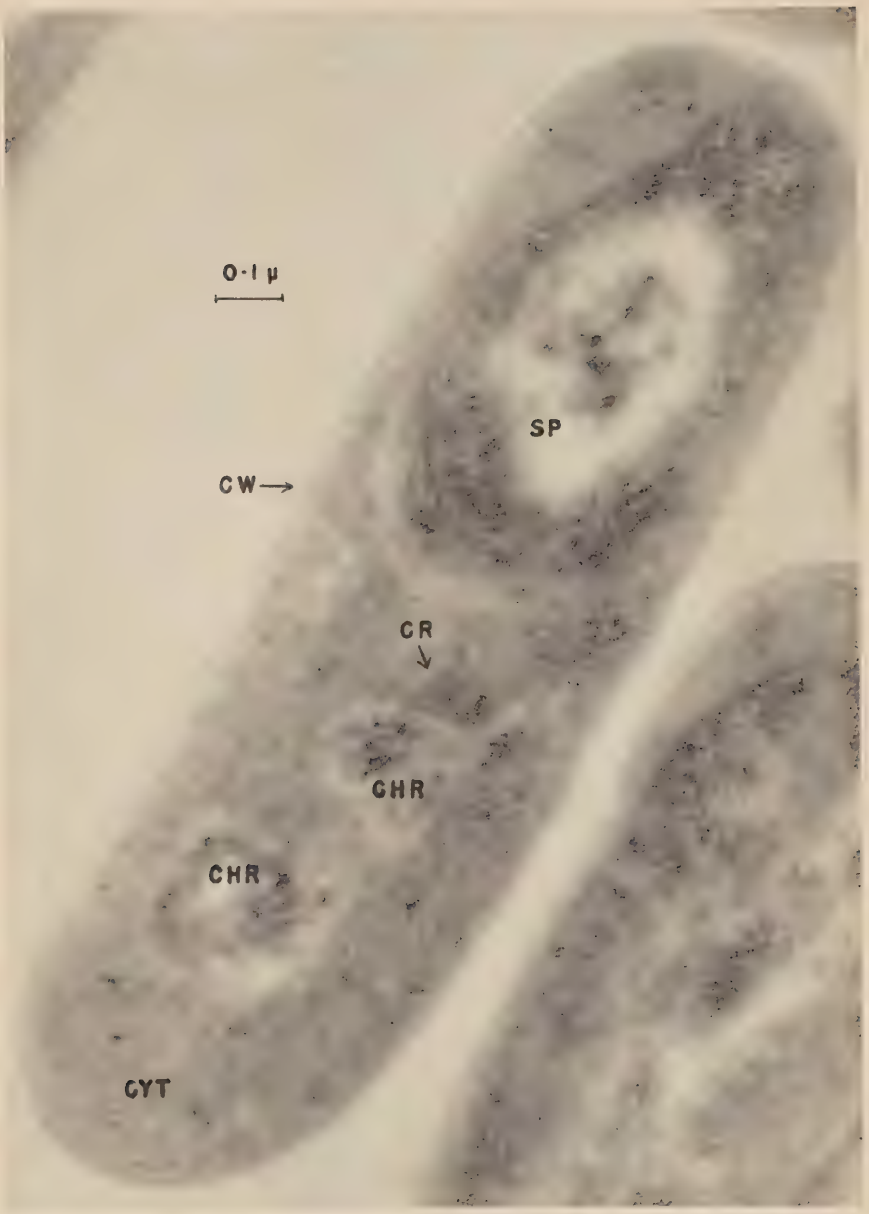

Figure 4—An electronmicrograph of an ultrathin section of a *Bacillus thuringiensis*. The cell was fixed and embedded for sectioning during the early stages of sporulation. The photograph illustrates the position of the developing protein crystalline toxin in relation to other cell components. The diamond-shaped crystal (cr) lies in the cytoplasm (cyt) between the spore (sp) and the chromatin bodies or nuclei (chr). The whole contents of the cell are enclosed within a cell wall (cw).

× 110,000

C. L. Hannay, unpublished work.

PLANT GROWTH CHAMBERS

The demand for experimental material, whether healthy, diseased or insect-infested plants, is so great and continuous that greenhouse facilities cannot meet it. An attempt is therefore being made to produce this material under standard and controllable conditions. For this purpose there has been erected a series of four growth chambers in which it is possible to control temperature, air-humidity and rate of flow, light intensity and day length. The value of this equipment for the production of experimental plants may well be eclipsed by its potential use as a research instrument.

The four chambers have been built below ground, each with a light section 12′ x 14′, a dark section 3′ x 11′, and an entrance corridor 3′ x 3′ to the dark section. The light section is lined with white structural glass, the dark section with black glass. Foam-glass insulation separates this glass from the outer cement walls. The floor of each chamber is of 4″ steel channels with a $\frac{1}{4}$″ slot between, and below this floor there are four air diffusers in a space 3′ high. The ceiling of each chamber, 7′ above the false floor, is of water-white plate glass, above which is a light loft maintained at 70°F., the optimum temperature for the operation of the slim-line fluorescent lamps. The light intensity obtained with a 1:4 wattage ratio of incandescent-fluorescent lamps is about 2500 ft-c. at plant level. The ballasts for the fluorescent lights are outside the rooms. In addition to the lights above glass, a small light panel 3′ x 8′, which can be raised or lowered by a winch, is placed inside each light chamber.

Provision is made for a range of 30 to 130 changes of air per hour, the highest air change representing a linear movement upwards of about 16′ per minute. This air is continuously circulated from the air-conditioning unit through the chamber; from 0 to 20 per cent of the air can be exhausted and replaced by a similar amount of preconditioned fresh air. In the conditioner the air passes over a large water-sprayed coil, the temperature of which is regulated by the humidity controller; it then passes through water-eliminators to an upper coil, the temperature of which is determined by the temperature controller. The treated air is forced by a fan through the diffusers into the plenum beneath the false floor of the growth chamber, up through the growing area, and is returned to the conditioner through duct openings along one wall of the room just below the light loft. The exhaust duct and the fresh air supply duct are joined to this return duct.

The two coils of each conditioner are supplied with hot and cold ethylene glycol-water, admitted by valves and controlled in amount requisite for the temperature required, and kept continuously circulating by means of a pump and by-pass system. The hot and cold ethylene glycol lines are supplied from mains, the former held at 180°F. by means of a steam convertor, the latter held at 30°F. by a refrigeration system of two compressors, two chillers and an evaporative condensor.

The temperature range within the growth chambers is from 40°-90°F. with a control of $\pm$ 0·5°F.; the humidity range is from 50-90 per cent relative humidity within this temperature range.

Provision is made for the automatic programing of temperature and humidity within these limits. The process may be described by outlining a day-night program requiring a day temperature of 75°F. and a night temperature of 55°F. A time "soaker" (clock) maintains the 75°F. temperature and, at the end of ten hours, a rate changer comes into operation, causing a fall of temperature of 5°F. an hour to 55°F. which is held by a second time-soaker. After six hours a second rate changer brings about a rise of temperature of

5°F. per hour to 75°F. which is maintained for ten hours. A similar process is carried through to produce the periodic change of relative humidity. A third rate changer to each set of temperature and humidity controls of the four chambers permits an elaboration of such a program. The actual temperature and humidity recorded by sensing elements in each chamber determines the addition of hot or cold glycol necessary to bring the figure to that required by the program, and these figures are graphed for permanent record.

The influence of artificial light on plant growth:

A necessary preliminary to the erection of the plant growth rooms was to ascertain which type of lamp or combination of types is most favorable to plant growth under controlled conditions. The study up to the present time has been confined to fluorescent lamps and fluorescent-incandescent mixtures. Three types of fluorescent lamps, white, daylight and standard cool white have been tested. In addition to this, tests have been carried out on a white fluorescent-incandescent mixture, and a standard cool white fluorescent-incandescent mixture.

The light panel consisted of 18 T8, 96″ fluorescent lamps on 2″ centers. The incandescent light used in the mixtures was supplied by 8 100-watt neck-reflector lamps on 24″ centers, 6″ above the fluorescents. Light intensity at first leaf height was slightly above 800 ft-c. under the fluorescent alone, and the incandescent lamps added 100-110 ft-c. to this total.

The spectra of the different light sources were photographed through a constant deviation spectrometer and compared, on a recording spectrophotometer, with the spectrum of a standard lamp photographed through a varying density wedge. Relative energy values for specified wave lengths were thereby obtained.

Beans were grown beneath the light panel on a 16-hour light, 8-hour dark regime through the vegetative period. Twenty plants were sampled at 160, 208, 256 and 304 hours of light from the straightening of the hypocotyl hook. After drying in a press the leaves were blueprinted, and with the leaf areas obtained from the blueprints and the dry weights, net assimilation rates were calculated.

During the 208-256 hour sampling period under the three types of fluorescent lamp, a drop occurred in the net assimilation rate. The addition of incandescent to the fluorescent resulted in an increase in net assimilation rate over the same sampling period. Apparently the plant dry weight did not increase in proportion to the increase in leaf area during this sampling period under fluorescent alone, but was increased by the addition of incandescent. For the 160-208 and the 256-308 hour sampling periods there was little difference in the net assimilation rates under the three types of fluorescent lamps or the two fluorescent-incandescent mixtures.

The results of the light analysis indicated that there is a difference in emitted energy for each type of fluorescent lamp. This difference occurs in the near red portion of the spectrum and has little or no effect on the assimilation rates of the plants. The addition of incandescent to the spectrum caused a marked increase in the total emitted energy in the red and near red portions of the spectra.

It is concluded that the increase in net-assimilation rate and dry weight of plants grown under the fluorescent-incandescent mixtures can be attributed to an increase in photosynthetic activity. This in turn may be attributed to either the increase in total emitted light energy or to the addition of energy in the red region of the spectrum. It is proposed to add an equal amount of energy to the blue part of the spectrum by the use of (colored) fluorescent lamps in order to determine which of the above propositions is correct.

CO-OPERATIVE WORK

Plant Pathology Laboratory, Vancouver:

The symptoms of many virus diseases resemble those produced by the application of plant growth substances, for example, the increased rooting of cuttings, lateral bud proliferation and parthenocarpic fruits of the tree tomato (*Cyphomandra betacea* Sendt.) infected with the witches broom virus of potato. An attempt is being made to obtain biochemical evidence of the auxin status of normal and virus-infested tree tomatoes.

Forest Biology Division and Ontario Dept. of Lands and Forests:

The accidental distribution of the European pine shoot moth (*Rhyacionia buoliana* Schiff.) on nursery stock is a serious hazard which may perhaps be reduced by the fumigation of shipments from infested nurseries. This possibility is being investigated. Ethylene dibromide is about six times as toxic as methyl bromide to the third- and fourth-instar larvae of this insect, the stage normally present in the stock when shipped. For field fumigation of seedlings heeled-in or piled for shipment under plastic gas-proof tarpaulins, however, methyl bromide is more convenient to use; having a lower boiling point (39°F.), it is more readily volatilized under the sheets.

Fumigation is carried out in early or mid-spring while the pine seedlings are still dormant. At this stage all four pine species concerned—Scots, red, white and Mugho pine—are tolerant to those dosages of both fumigants that are lethal to the third- and fourth-instar larvae of the pine shoot moth. At stages later than dormancy, the pine seedlings may be more susceptible to damage by the fumigant, and this damage is now being studied.

Forest Biology Division:

The investigation of many problems in the formulation of spray concentrates, and in the construction of spraying machines, requires methods for the production of spray deposits of known uniform droplet size. Mr. A. C. Rayner of the Forest Biology Division devised an apparatus suitable in the range of droplets of 100-500 microns MMD. A settling tower has been constructed for the preparation of deposits of droplets of diameter down to 20 microns MMD, and by means of this apparatus and Rayner's apparatus spray deposits of aqueous suspensions of DDT have been prepared. The bioassay data using *Musca domestica* indicate that the LD_{50} of deposits of droplets around 200 microns MMD is three to four times greater than that of droplets of around 20 microns MMD. The latter droplets yield a steeper regression line of probit-mortality on the logarithm of DDT concentration.

Ontario Department of Agriculture:

The wax moth *Galleria mellonella* L., a serious pest of honey combs in storage, has been successfully controlled for the past 15 years by fumigation with methyl bromide. In view of legislation by the Province of Ontario prohibiting the application of this fumigant by any but licensed fumigators, a request was made by the Ontario Department of Agriculture for a suitable and effective fumigant to replace it. In collaboration with the Departments of Entomology and Apiculture at the Ontario Agriculture College, Guelph, experiments were made on all stages of *G. mellonella* in comb honey. Acrylonitrile was found to be a good substitute, as it was toxic to all stages of the insect at suitable dosages. In practice it is necessary to apply this chemical in admixture with carbon tetrachloride. In the course of this investigation the eggs were fumigated at four different ages. The mortalities at these four stages of development appeared to follow different patterns which were distinctive of

the fumigant, whether aliphatic halide, epoxide, nitrile or chlorinated nitroparaffin. Acrylonitrile meets the requirements of the Ontario Department of Agriculture, for, though poisonous to man, it has a warning odor.

Botany Division:

An important quarantine problem is the possibility of the spread of unwanted pathogenic organisms in secondhand jute bags. There is a lively trade in this commodity and shipments are moved from one agricultural area to another. This material is known to carry *Corynebacterium sepedonicum* (Spiek. & Kotth.) Skatterson & Burkholder, causing bacterial ring rot of potatoes. Vacuum fumigation with ethylene oxide has been shown of promise as a method of destroying the organism in bales of jute bags. As vacuum fumigation facilities are not generally available in Canada, a study has been undertaken of the possibility of control under atmospheric conditions.

Harrow Science Service Laboratory:

The work at the Harrow Laboratory has shown that ethylene dibromide gives erratic results as a soil fumigant on certain soils, and it was suspected that the cause is adsorption of the former fumigant by soil colloids, a possibility which we were asked to investigate. In the preliminary work on the estimation of ethylene dibromide it was found that the concentration of ethylene dibromide in the free space of the fumigation chamber was not in direct proportion to the dose used but decreased at lower dosages in a manner suggestive of an adsorption of the fumigant on the walls of the chamber.

Entomology Division—Fruit Insect Unit:

The effective use in Nova Scotia and elsewhere of ryania for the control of codling moth without deleterious effect on beneficial insects has led, in addition to the studies of its insecticidal components outlined in page 20, to a search for methods of reducing spray deposit. It has been found in British Columbia that the heavy residue left on apple by sprays containing 6 lb. ryania per 100 gallons results in a poor development of fruit color and size. Various extracts and formulations of ryania have been prepared and submitted for field assay.

D.S.I.R. Pest Infestation Laboratory, Slough, England:

Because no suitable large vacuum fumigation chamber was available at the Pest Infestation Laboratory for the examination of the new technique of "atmospheric pressure restored" fumigation (see page 29) devised by Burns, Brown and Heuser of that institution, tests on a commercial scale were carried out at the Montreal Fumigation Station. The new method was most successful in killing insects within the bales, but it was found that fumigant concentration and insect kill near the surface of the bales were weak (29).

Department of Botany, University of Western Ontario:

Arising from the work reported on page 24 of the effects of certain substituted ureas and pseudoureas on root growth, Dr. D. A. McLarty, of the University of Western Ontario, undertook a cytological study of the affected rootlets. The effects on corn root tips of 3:3-di(*p*-chlorophenyl)-2-methyl pseudourea were compared with those of the herbicides CMU (3-(*p*-chlorophenyl)-1:1-dimethyl urea) and dichloral urea. All three chemicals cause some disruption of the surface cells, but the pseudourea also affected the vascular tissue. With all three compounds mitosis was suppressed coincident with the suppression of the root extension.

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